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AB Endothelial cell migration is a key feature of angiogenesis. Epidermal Growth Factor (EGF) or Tumor Angiogenesis Factor (TAF) induce cell migration and angiogenesis. When the matrix components, collagen or fibronectin, were used as a substratum in the phagokinesis **assays**, EGF- or TAF-induced cell migration was inhibited. It has been proposed that TAF activates cellular protease causing the matrix degradation that is evident during neovascularization in vitro. If such degradation leads to cell migration and angiogenesis, then other agents that interfere with the synthesis or assembly of matrix components should stimulate cell migration and angiogenesis. The proline analogues **cis hydroxyproline**, azetidine and dehydroproline are known modulators of cellular collagen synthesis. At optimal concentration (10(-5)M) these analogues caused 3-fold increases in endothelial cell migration rates in vivo as tested by a subcutaneous implant **assay**. We conclude from these studies that: (i) matrix components control cellular migration rates; high concentration of collagen or fibronectin inhibit angiogenically active inducers of endothelial cell migration. (ii) Intracellular modulation of synthesis of collagens leads to angiogenesis by stimulating cell migration. These findings relate to tumor angiogenesis and that TAF might trigger angiogenesis either by activation of latent proteases or by some modification of matrix assembly during synthesis that affects cell adhesion and migration.

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MATRIX CONTROL OF TUMOR ANGIOGENESIS

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INTRODUCTION

Tumor angiogenesis is the process through which certain tumors stimulate the growth of the microvascular network in the surrounding tissue. This capillary network is remarkable in that the growth is directed towards the tumor which becomes vascularized. An important fundamental question is, what is the nature of the molecular controls responsible for the directed vascularization? As cell migration is the salient feature in neovascularization the question thus becomes, what are the molecular events that control cell migration during neovascularization? A wide variety of compounds have been shown to be inducers of angiogenesis or neovascularization *in vivo*. These include both tumor or tissue derived factors as well as a number of chemical factors.[1,2,3,4,5,6] All of these, with the exception fo angiogenin, have been shown to also induce endothelial cell migration.

Clearly cell migration *in vivo* must require the modification of a number of cellular processes together with changes in the physical environment before a cell can escape from the matrix in which it is embedded. This matrix modification occurs during angiogenesis and is reported to influence cell migration and proliferation.[7,8,9] Rifkin et al. (1983)[10] proposed that during angiogenesis endothelial cells release specific proteases to help them escape matrix constraints and penetrate the surrounding stroma. Likely candidates are plasminogen activator and collagenase. These proteases were shown to be at much higher levels after exposure of the cells to angiogenesis factors. This was in accord with the idea that extracellular matrix (ECM) disruption was a likely early event in angiogenesis. ECM has been also shown to have a profound influence on the early events in organogenesis and development and has been reported to have an effect on both cell proliferation and migration.[11,12,13] These studies and others have shown ECM to not only affect the direction of migration but also the rate at which cell migration occurs.[12,14,15]

In our attempts to focus on endothelial cell migration as the major controlling event in angiogenesis, we have examined how ECM components influence cell migration and angiogenesis. In this paper we demonstrate that (i) ECM components control cellular migration rates by inhibiting the migration inducing activity of angiogenic factors and (ii) interference with ECM synthesis or assembly stimulates cell migration which leads to angiogenesis.

MATERIALS AND METHODS

Cell Cultures

All cells were of bovine origin. Clonal lines of aortal endothelial cells (BAE), retinal capillary endothelial cells (BREC), smooth muscle cells (BSM) and corneal endothelial cells (BCE) were established and maintained as described (McAuslan et al., 1987). All cells used for experimentation were between their 6th and 12th passage.

Migration Assay

Cell migration was determined using the phagokinesis assay.[16] Migration inducers were added to the medium covering the cells and track lengths measured 24 hours later. Measurements were made using a Leitz Bioquant II image analysis system. An average of 100 individual tracks were measured and the means tabulated.

Substratum Preparation

Serial dilutions of fibronectin (FN) and collagen were prepared using serum free medium. Two millilitre aliquots of each concentration in triplicate were added to 60 mm tissue culture dishes containing clean 18 mm glass coverslips. These were then air dried. Two millilitres of colloidal gold solution were then added and incubated at 37°C for 2 hours. The excess liquid was then removed and 5 ml of migration medium containing 3% FCS and 3×10^4 cells added and reincubated.

Reagents

The proline analogues cis-4-hydroxy-L-proline (cisOHPro), 3,4 dehydro-L-proline (dHPRo), cis-4-hydroxy-D-proline (cisDPro) and L-azetidine—Z-carboxylic acid (Azet) were obtained from Sigma Chemical Co., USA. Fibronectin was isolated from fresh bovine plasma using the method of Hannan et al. (1984).[17] Collagen as Vitrogen 100 was purchased from The Collagen Corporation, Palo Alto, CA and is a purified form of type I collagen from calf skin. EGF was prepared from acid extracts of mouse submaxillary glands and was further purified by reverse phase HPLC.[18] ESF was prepared according to McAuslan and Hoffman (1979).[19]

In vivo Angiogenesis Assay

Slow release Elvax polymer was impregnated with saturating amounts of test material. These were sterilized and embedded in an atelo-collagen gel in a shallow silicon tube. These were then implanted subcutaneously into rabbits and examined 10 days later.[20] The implants were then surgically removed, fixed in normal saline and processed for light microscopy.

RESULTS

Changes in the Rate of Migration of Endothelial Cells in Response to Angiogenic Factors: Inhibition by ECM Components

The migratory response of endothelial cells to angiogenic factors has been well documented.[1,21,22] Two of these factors, EGF and ESF, were used to examine the possible inhibition of endothelial cell migration by the ECM components fibronectin (FN) and collagen. Bade and Nitzgen (1985)[15] have shown that FN, when bound to the substratum, inhibited the migration of Buffalo rat liver epithelial cells in response to a combination of EGF and insulin. This inhibition was shown to be FN specific and they suggested that it may be an additional control mechanism to contact inhibition.

Our approach was to examine endothelial cells for similar mechanisms by using angiogenic factors that are known to produce large increases in endothelial cell migration. The two factors, EGF and ESF, gave consistently 3-fold increases in the migration rate of the different endothelial cells tested, using BSA as the substratum for the attachment of the colloidal gold particles, and were active over a large concentration range (Table 1). However, when either of the two factors were used at optimal concentrations normally producing maximal migration, inhibition of the migration occurred if FN was used as a substratum.

A series of concentrations of FN were then tested to determine the concentration at which total inhibition occurred. Table 2 presents these results. It was found that both EGF- and ESF-induced migration was inhibited linearly as the FN concentration increased. Complete inhibition occurred at concentrations above 30 $\mu g/cm^2$. To ensure that the observed migration inhibition was substratum dependent a similar series of concentrations of FN were added to the medium in a set of experiments where BSA was used as the substratum. No inhibition of the EGF- or ESF-induced migration was observed under these conditions.

We next examined collagen type I substratum for its ability to inhibit migration as did the FN substratum. Bade and Nitzgen (1985)[15] had tested collagen type IV at a concentration simi-

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Table 1. Stimulation of BREC migration by EGF or ESF on BSA substratum

BSA conc. $\mu g/cm^2$	EGF conc. ng/ml	Area $10^{-3}\mu m^2$	ESF conc. $\mu g/ml$	Area $10^{-3}\mu m^2$
3000	100	96.2	50	122.3
3000	50	75.4	25	65.8
3000	25	58.8	10	36.5
3000	10	42.7	0	26.8
3000	0	29.4	–	–

BREC were seeded onto BSA coated coverslips in Medium 199 plus 3% FCS and incubated at 37°C in 5% CO_2 for 24 hours. The migration trails were then measured using a BIOQUANT image analysis system. Each measurement represents the mean of 100 individual migration trails.

Table 2. The effect of fibronectin substratum on the stimulation of BREC migration by EGF or ESF

FN conc. $\mu g/cm^2$	EGF conc. ng/ml	Area $10^{-3}\mu m^2$	ESF conc. $\mu g/ml$	Area $10^{-3}\mu m^2$
0.0	100	86.9	50	97.3
0.3	100	88.7	50	92.8
3.0	100	47.8	50	48.5
30	100	29.9	50	30.8
60	100	25.7	50	26.8

BREC were seeded onto FN coated coverslips in Medium 199 plus 3% FCS and incubated at 37°C in 5% CO_2 for 24 hours. The migration trails were then measured using a BIOQUANT image analysis system. Each measurement represents the mean of 100 individual migration trails.

Table 3. The effect of collagen substratum on the stimulation of BREC migration by EGF or ESF

Collagen $\mu g/cm^2$	EGF conc. ng/ml	Area $10^{-3}\mu m^2$	ESF conc. $\mu g/ml$	Area $10^{-3}\mu m^2$
0.0	100	89.9	50	99.7
3.0	100	86.7	50	96.8
30	100	75.5	50	78.5
60	100	62.9	50	65.1
100	100	24.4	50	23.4

BREC were seeded onto collagen coated coverslips in Medium 199 plus 3% FCS and incubated at 37°C in 5% CO_2 for 24 hours. The migration trails were then measured using a BIOQUANT image analysis system. Each measurement represents the mean of 100 individual migration trails.

lar to that of FN used in these experiments and found it to have no effect. We found that collagen type I did inhibit EGF- and ESF-induced migration but at a concentration an order of magnitude greater than that required by FN (Table 3). The high concentration of collagen needed for inhibition to occur could explain why it was missed by Bade and Nitzgen. Alternatively, it is conceivable that collagen type IV does not have any effect. It will be necessary to repeat this work with collagen type IV to resolve this.

We attempted to demonstrate further that the ECM components already shown to inhibit

Table 4

Cell type	Conc. of analogue	Area			
		cisOHPro	cisDPro	Azet	dHPro
BREC	5×10^{-4}M	20.0	23.2	19.9	21.0
	1×10^{-4}M	29.2	25.2	30.1	30.5
	1×10^{-5}M	46.4	24.2	37.9	48.1
	1×10^{-6}M	24.2	24.7	25.0	25.0
BCE	5×10^{-4}M	30.0	29.8	29.5	30.5
	1×10^{-5}M	115.8	36.8	43.4	45.1
	1×10^{-6}M	36.4	38.6	43.4	45.1
BASM	1×10^{-5}M	89.4	33.6	110.6	90.8

Cell migration rates in response to proline analogues. Each measurement represents the mean of 100 cell trails. The measurements were made with a BIOQUANT II analysis system.

the migration of endothelial cells was not just a response to high concentrations of proteins on the substratum. A similar series of experiments using ovalbumin or BSA as the substratum protein were carried out. It was found that neither ovalbumin nor BSA also had any effect on induced migration over the concentration range tested. This suggests that specific ECM components may act as molecular modulators regulating cell migration. The ECM must therefore be degraded or modified for endothelial cell migration to occur during neovascularization.

Stimulation of Endothelial Cell Migration via Interference with ECM Synthesis

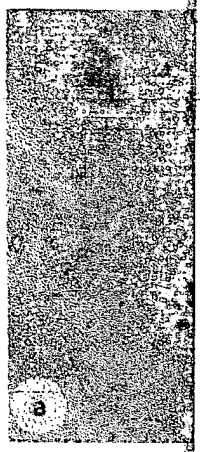
It has been shown that tumor angiogenic factor (TAF) activates a latent collagen type IV collagenase.[23] Further, it has been demonstrated that endothelial cells migrating in response to the angiogenic stimulus of retinal extracts actively degrade basement membrane collagens.[24] Angiogenic factors therefore may stimulate ECM degradation by either (1) activating collagenases produced by endothelial cells which in turn degrade the ECM to which they are adhering, or (2) cause some change in the synthesis or export of ECM components which in turn leads to increased enzymatic degradation and migration occurs.

In either case, and in the light of the previously reported ECM inhibition results, it should be possible to induce endothelial cell migration by modifying synthesis of certain ECM components, in particular the collagens, by the use of proline analogues. A range of concentrations of cisOHPro, cisDPro, Azet and dHPro were tested using the previously described assay with BSA as the substratum. All the analogues interfere with the synthesis and secretion of collagens. The results presented in Table 4 show cisOHPro, Azet and dHPro all produce a 2-3 fold increase in migration rate; cisDPro, which is not incorporated into collagen, was inactive. The optimal concentration was 10^{-5}M; concentrations above this produced a relative decrease in migration rate. This later finding agrees with the work of Madri and Stenn (1982).[14] They found that marked inhibition of collagen synthesis with proline analogues inhibited migration, suggesting that ECM turnover by migrating endothelial cells is necessary for continued migration.

Proline Analogues as Angiogenic Factors in vivo

It has become evident that any factor that induces endothelial cells to migrate *in vitro* is invariably angiogenic *in vivo*. We demonstrated this by implanting slow release polymers subcutaneously in rabbits.[20] These implants were removed after 10 days, fixed, processed histologically and examined microscopically. A typical field is presented in Figure 1.

Control implants showed responses that were consistent with those of a foreign body. There was an increase in granulocytes at the edge of the silicon ring with an increase in fibroblasts and the appearance of dense collagen bundles. On the other hand, implants containing the proline analogues were markedly different. These were highly vascularized with numerous large and small vessels.



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DISCUSSION

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ECM Synthesis

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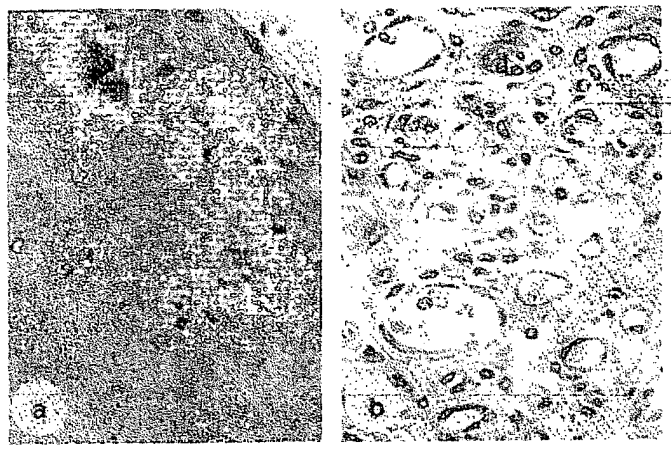

Fig. 1. (a) Control implant in collagen gel at 10 days, (b) cisOHPro implant in collagen gel at 10 days.

DISCUSSION

Extracellular matrix (ECM) has been shown to influence profoundly the behaviour of vascular cells; it plays a role in directing cell migration during development and wound healing and affects the action of soluble growth regulators.[12] The endothelial cell in turn also influences the ECM during migration, continually modifying it through the action of proteases.[25] The experiments reported above attempt to further examine these ECM-endothelial cell interactions during migration by (1) demonstrating that the ECM components fibronectin and collagen I inhibit the migration response to the angiogenic factors EGF and ESF, and (2) modulation of the synthesis of ECM collagen is sufficient to induce endothelial cell migration *in vitro* and set angiogenesis en train *in vivo*. The ability of ECM to inhibit the action of angiogenic factors is of general interest. Bade and Nitzgen (1985)[15] proposed the inhibition they observed had relevance to liver physiology as the cells used were liver epithelial cells, but these results would indicate that this control mechanism is yet another general function of the ECM.

We found that the inhibition of endothelial cell migration was proportional to the concentration of the ECM component. The maximal inhibition occurred at 30 $\mu g/cm^2$ and 100 $\mu g/cm^2$ for FN and collagen respectively. As the concentration on the substratum decreased the induction of migration by EGF or ESF increased, indicating a linear relationship for the effect. Addition of FN at a concentration of 60 $\mu g/cm^2$ to the medium in soluble form rather than as a substratum did not inhibit migration to EGF or ESF (data not presented). It is conceivable that if this effect operated *in vivo* that a factor like TAF which has been reported to activate a latent protease[25] could produce a concentration gradient which would directionally control the migration of endothelial cells during angiogenesis.

Further, our finding that the modification of ECM through the modulation of synthesis of cellular collagens leads to migration and angiogenesis supports the hypothesis that the early event in angiogenesis is matrix modification which in turn controls endothelial cell migration and directional movement. The proline analogues used to interfere with collagen secretion and synthesis, cisOHPro, Azet and dHPro, all produced an increase in the cell migration rate when used at their optimal concentrations 10^{-5}M. This produced a 2-3 fold increase over the control. Since cisDPro was inactive this suggests a structural specificity. Continuous collagen synthesis has been shown to be an obligatory requirement for cell migration.[14] Our findings suggest that small changes in the collagen structure due to the incorporation of low levels of proline analogues is sufficient to impair the assembly of collagen in the ECM. This may destabilize cell anchorage, interfere with some other step critical for cell migration or perhaps make the ECM more susceptible to degradation, with the result being an increased migration rate.

The finding that modulation of collagen synthesis or secretion can lead to altered controls on the microvascular system offers a useful model system to understand the cellular events in tumor angiogenesis in particular and organogenesis in general.

SUMMARY AND CONCLUSION

Endothelial cell migration is a key feature of angiogenesis. Epidermal Growth Factor (EGF) or Tumor Angiogenesis Factor (TAF) induce cell migration and angiogenesis. When the matrix components, collagen or fibronectin, were used as a substratum in the phagokinesis assays, EGF- or TAF-induced cell migration was inhibited. It has been proposed that TAF activates cellular protease causing the matrix degradation that is evident during neovascularization *in vitro*. If such degradation leads to cell migration and angiogenesis, then other agents that interfere with the synthesis or assembly of matrix components should stimulate cell migration and angiogenesis. The proline analogues cis hydroxyproline, azetidine and dehydroproline are known modulators of cellular collagen synthesis. At optimal concentration (10^{-4}M) these analogues caused 3-fold increases in endothelial cell migration rates *in vivo* as tested by a subcutaneous implant assay. We conclude from these studies that: (i) matrix components control cellular migration rates; high concentration of collagen or fibronectin inhibit angiogenically active inducers of endothelial cell migration. (ii) Intracellular modulation of synthesis of collagens leads to angiogenesis by stimulating cell migration.

These findings relate to tumor angiogenesis and that TAF might trigger angiogenesis either by activation of latent proteases or by some modification of matrix assembly during synthesis that affects cell adhesion and migration.

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